Protein and vesicle dynamics in regulated secretion

Cijsouw, A.

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F(t)-F(t bleach)
F(t pre-bleach)-F(t bleach)
q =
h
s ~ 10 m
v ~ 5 m
x' = x0 + mt'
y' = y0 + nt'
z' = z0 + pt'
Formula Ross's
5) v = 2πr / T
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Role of FEZ1 in transport of syntaxin-1 vesicles in developing hippocampal neurons

ABSTRACT

Syntaxin-1 is a trans-membrane protein essential for synaptic transmission. Recently, syntaxin-1 was found to interact with the kinesin-1 adaptor protein, FEZ1, in a phosphorylation-dependent manner in C. elegans. To investigate the role of FEZ1 in transport of syntaxin-1 in mammals, we expressed syntaxin-1-EFYP in developing hippocampal neurons and developed a novel method to discriminate vesicular from plasma membrane bound syntaxin-1-EYFP in living cells. We show that during development, neurons express syntaxin-1 at vesicles and at the plasma membrane. Syntaxin-1 vesicles were found in dendrites and axons and primarily engaged in bidirectional transport. Transport appeared to be independent of the phosphorylation status of FEZ1. This suggests that phosphorylation of FEZ1 does not play an important role in transport of vesicular syntaxin-1 in developing hippocampal neurons.

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5.1 INTRODUCTION

A multi-subunit SNARE-protein complex and SM (Sec1/Munc18-like) proteins, controlled by a myriad of other proteins, execute intracellular membrane fusion (Brunger, 2005; Jahn and Scheller, 2006). The SNARE-protein complex responsible for Ca2+-triggered synaptic vesicle fusion and consequent release of neurotransmitters into the synaptic cleft consists of vesicular SNARE protein synaptobrevin-2/VAMP2 and target SNARE proteins SNAP25 and syntaxin-1 (Jahn and Fasshauer, 2012; Rizo and Südhof, 2012). Syntaxin-1A is abundantly expressed at the plasma membrane of most neuron types of the brain (Inoue and Akagawa, 1993; Inoue et al., 1992) and may be functionally redundant with its homologue syntaxin-1B (Fujiwara et al., 2006; Gerber et al., 2008). Syntaxin proteolysis by botulinum neurotoxin type C1 inhibits neurotransmission (Blasi et al., 1993) and catecholamine release (de Wit et al., 2006), suggesting an essential role in vesicle fusion. Disruption of syntaxin-1 may be related to human neuropsychological alterations (Beasley et al., 2005; Brose, 2008; Feng et al., 2005; Meyer-Lindenberg et al., 2006; Nakamura et al., 2008; Nakayama et al., 1998; Wong et al., 2004) and behavioral dysfunction related to impaired synaptic plasticity in mice (Fujiwara et al., 2006; Fujiwara et al., 2010; Mishima et al., 2012). Hence, syntaxin-1 is required for synaptic vesicle fusion and normal brain functions. However, the molecular mechanisms that control transport of syntaxin-1 are largely unknown.

Syntaxin-1 is composed of an N-terminal α-helical domain (the Habc domain), and a C-terminal SNARE motif and trans-membrane region. Before SNARE complex formation, syntaxin-1 is found in a 'closed' conformation, in which the Habc domain is bound to the SNARE domain, and only binds to Munc18-1 (Dulubova et al., 1999; Misura et al., 2000). This syntaxin-1/Munc18-1 complex may stabilize both proteins, prevent premature SNARE complex formation and/or support its transport to the plasma membrane (McEwen and Kaplan, 2008; Medine et al., 2007; Rowe et al., 2001; Verhage et al., 2000; Voets et al., 2001; Zhou et al., 2013). Although Munc18-1 is not essential for syntaxin-1 targeting to the presynaptic terminal (Toonen et al., 2005). After arrival at the presynaptic terminal, the syntaxin-1/Munc18-1 dimer may function as starting point for SNARE complex formation (Jahn and Fasshauer, 2012; Ma et al., 2012; Rizo and Südhof, 2012).

The type II trans-membrane protein syntaxin-1 is inserted into the endoplasmic reticulum after translation on free ribosomes (Burri and Lithgow, 2004) and transported through the Golgi apparatus towards the plasma membrane. Munc18-1 may bind syntaxin-1 directly after translation allowing undisturbed trafficking of syntaxin-1 through the Golgi and to the plasma membrane (Liu et al., 2004; McEwen and Kaplan, 2008; Medine et al., 2007; Rowe et al., 2001; Rowe et al., 1999). In mature neurons syntaxin-1 is mainly transported through the axon via lateral diffusion (Mitchell and Ryan, 2004; Ribrault et al., 2011). In developing hippocampal neurons, syntaxin-1 is transported on vesicles via binding to kinesin adaptor protein syntabulin (Su et al., 2004), which interacts with motor protein kinesin family member 5 (KIF5). In a recent study it was found that syntaxin-1 is present in Munc18-1/FEZ1/kinesin transport complexes from rat brain and co-localizes in puncta with FEZ1 in growth cones of developing hippocampal neurons, suggesting that FEZ1 is a second adaptor protein for syntaxin-1 transport in vertebrates (Chua et al., 2012). In addition, binding of FEZ1 to kinesin-1 and Munc18-1 is regulated by phosphorylation of FEZ1, with a conserved site (serine 58) essential for binding. Expression of a phosphorylation-deficient mutant FEZ1 is unable to restore axonal transport of syntaxin-1 in Fez-1 deficient neurons in C. elegans, suggesting that FEZ1 phosphorylation is required for syntaxin-1 transport in C. elegans (Chua et al., 2012). However, it is unknown if phosphorylation of FEZ1 serine 58 plays a role in developing hippocampal neurons.

Here, we studied the transport of syntaxin-1 in developing hippocampal neurons and the role of FEZ1 S58 phosphorylation in this process. We expressed syntaxin-1-EYFP in dissociated hippocampal cultures and devised a novel method that simultaneously quenches plasma membrane syntaxin-1-EFYP and de-quenches vesicular syntaxin-1-EYFP to visualize and track syntaxin-1-EFYP vesicles. We show that syntaxin-1-EFYP is expressed in developing neurons at the membrane of axons and dendrites and acidic transport vesicles within these neurites. In addition, we found that these vesicles also transport synaptophysin-mCherry. Syntaxin-1-EFYP vesicles are mobile and in majority display bidirectional transport. However, syntaxin-1-EFYP transport was unchanged after expression of phosphorylation-deficient mutant FEZ1. This suggests that phosphorylation of FEZ1 on serine 58 does not play a major role in transport of syntaxin-1 in hippocampal neurons.

5.2 RESULTS

5.2.1 A NOVEL METHOD FOR VISUALIZATION OF SYNTAXIN-1-EYFP ON ACIDIC VESICLES

Syntaxin-1 is a type 2 trans-membrane protein expressed throughout the neuron (Garcia et al., 1995). Type 2 trans-membrane proteins have one membrane-spanning domain with their carboxyl (C) terminus located in the vesicle lumen or cell exterior and their amino (N) terminus on the cytoplasmic side. (Figure 5.1A, inset). We fused fluorescent enhanced YFP (EYFP) to the c-terminus of syntaxin-1 and expressed the fusion protein in cultured hippocampal neurons to visualize syntaxin-1 transport. Syntaxin-1-EYFP expressed at the plasma membrane is fluorescent, while syntaxin-1-EYFP on vesicles with an acidic lumen is not (Figure 5.1A, control application)(Mitchell and Ryan, 2004) due to the quenching of EFYP by protons (Jayaraman et al., 2000). Visualization of vesicular syntaxin-1-EYFP requires the quenching of plasma membrane expressed syntaxin-1-EYFP and de-quenching of vesicular syntaxin-1-EYFP. Fluorescent proteins exposed to the extracellular milieu can be quenched by cell impermeable bromophenol blue (BPB) (Harata et al., 2006), while fluorescent proteins exposed to an acidic milieu can be de-quenched by cell permeable ammonia (NH_3) in the form of dissolved NH_4Cl (pH 7.4) thereby neutralizing the pH of the vesicular lumen (Miesenböck et al., 1998). We combined both methods (Figure 5.1A, BPB/ NH_4Cl application) to simultaneously decrease fluorescence from plasma membrane syntaxin-1-EYFP with BPB (Figure 5.1A, blue gradient) and increase fluorescence from vesicular syntaxin-1-EYFP with NH_3(Figure 1A). In an imaging experiment, we first applied BPB to ensure complete quenching of plasma membrane syntaxin-1-EYFP before applying BPB/ NH_4Cl to visualize syntaxin-1-EYFP on vesicles (Figure 5.1B). Indeed, syntaxin-1-EYFP (Stx-YFP) expressed in cultured hippocampal neurons showed fluorescence throughout the neuronal processes (Figure 5.1C, 6 s), which was quenched by BPB (Figure 5.1C, 20 s). Fluorescence from vesicles appeared (Figure 5.1C, arrowheads) upon pH neutralization by addition of NH_4Cl (pH 7.4) (Figure 5.1C, 24 s). Vesicles were stationary or moved through the neuronal process (Figure 5.1C, compare closed with open arrowhead at 24 s, 38 s and 60 s). A kymograph analysis showed that multiple vesicles appeared after BPB/ NH_4Cl (pH 7.4) application and moved with different speeds through the neuronal process. Hence, the local application of bromophenol blue/NH_4Cl (pH 7.4) is a novel method to visualize syntaxin-1-EYFP transport vesicles.

5.2.2 SYNTAXIN-1-EYFP IS EXPRESSED ON PLASMA MEMBRANE AND VESICLES OF DEVELOPING NEURONS

Fluorescent syntaxin-1 displays lateral diffusion in the plasma membrane of mature hippocampal neurons in culture (Mitchell and Ryan, 2004; Ribrault et al., 2011)(Chapter 3 this thesis), and is transported on vesicles of ventral nerve cords in C. elegans (Chua et al., 2012) and cultured

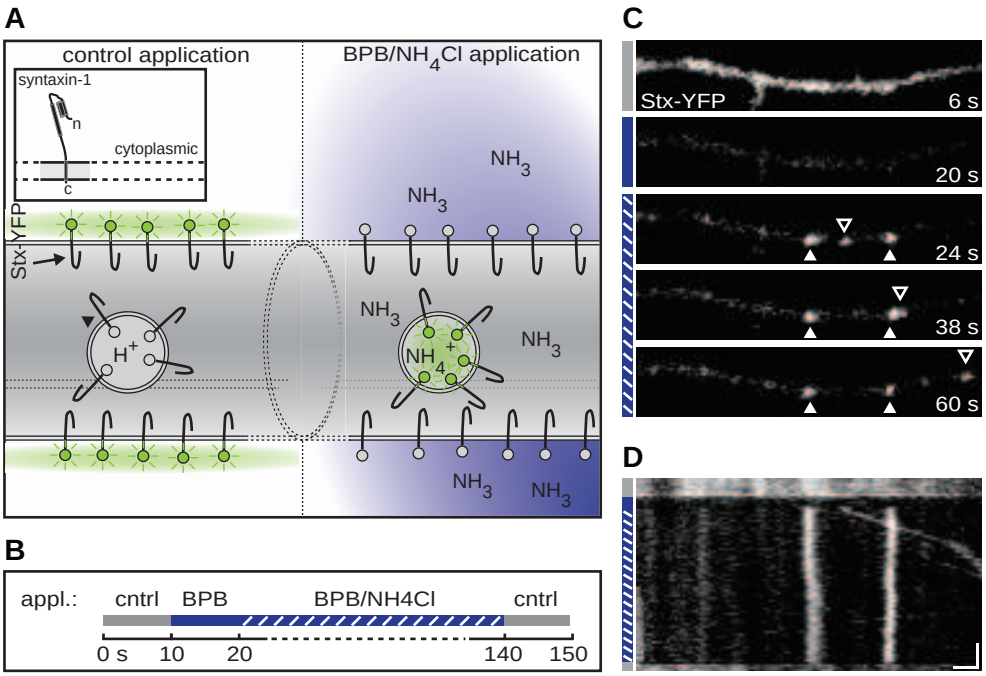


Figure 5.1 A novel method for visualization of syntaxin-1-EYFP transport vesicles A, Cartoon explaining how to visualize Syntaxin-1-EYFP (Stx-YFP) vesicles amidst plasma membrane expressed Stx-YFP. Syntaxin-1 c-terminus is located at the extracellular side of the plasma membrane (or in the lumen of vesicles) and the n-terminus at the cytoplasmic side (inset). C-terminal tagged EYFP of vesicular Stx (arrowhead) is quenched by protonation and c-terminal tagged EYFP of plasma membrane bound Stx (arrow) is the main source of fluorescence (Mitchell and Ryan, 2004). Hydrophilic bromophenol blue (BPB, blue gradient) is cell impermeable and quenches plasma membrane bound Stx-YFP. Cell permeable NH3, from NH4Cl (pH 7.4), neutralizes pH in vesicular lumen and increases fluorescence of vesicular Stx-YFP. Therefore, the main source of fluorescence during bromophenol blue/NH4Cl (pH 7.4) application is from neutralized intracellular compartments expressing Stx-YFP. B, Application protocol to visualize Stx-YFP: grey bar indicates control application (cntrl, Tyrodes solution), blue bar indicates BPB application and blue bar with white dashes indicates BPB/NH4Cl (pH 7.4) application. Imaging at 0.5 Hz. C, Example of Stx-YFP transport vesicles visualization. Stx-YFP in a neuronal process shows high overall expression during control application (6 s). With BPB superfusion (20 s) the majority of fluorescence decreases indicating that Stx-YFP is plasma membrane expressed. Upon additional NH4Cl (pH 7.4) fluorescent puncta appear (24 s, arrowheads) indicating neutralized vesicles expressing Stx-YFP. Vesicles can be seen moving through the neuronal process (38 and 60 s, open arrowhead). Colored bars besides panels indicate type of application (according to B). D, Kymograph analysis from neuronal process in C shows Stx-YFP vesicle traces. Column besides kymograph indicates application. Stx-YFP, syntaxin-1-EYFP; BPB, bromophenol blue; cntrl, control.

hippocampal neurons (Su et al., 2004). Stx-YFP expressed in developing hippocampal neurons in culture at 5 and 6 days in-vitro (DIV) was located in the perinuclear region, likely the Golgi apparatus, and throughout the neuron (Figure 5.2A). Fluorescence was most prominent at edges of neuronal processes (Figure 5.2A, arrows bottom panel) indicating expression of syntaxin-1-EYFP at the plasma membrane. Indeed, BPB/ NH_4Cl (pH 7.4) reduced overall fluorescence (Figure 5.2A, top panel) and specifically at the edges of neuronal processes (Figure 5.2, compare arrows in A and B). Additionally, BPB/ NH_4Cl (pH 7.4) visualized vesicles (Figure 5.2B, arrowheads) within the same neuronal processes (Figure 5.2C). Together, these results show that syntaxin-1-EYFP is expressed on the plasma membrane and acidic vesicles of developing hippocampal neurons in culture.

5.2.3 VESICULAR SYNTAXIN-1-EYFP CO-LOCALIZES WITH SYNAPTOPHYSIN-MCHERRY AT AXONS AND DENDRITES

Syntaxin-1 vesicles co-transport active zone (AZ) constituents to new synapses in developing hippocampal neurons (Cai et al., 2007) and are located at growth cones of axons (Chua et al., 2012). To understand the targeting of syntaxin-1-EYFP, we examined the morphology of developing neurons in culture. Developing neurons in culture are characterized by one extended and several short processes representing the axon and dendrites, respectively (Dotti et al., 1988). Stx-YFP puncta (Figure 5.3A, arrowheads) were found both in axons and in dendrites. Stx-YFP fluorescence was higher in the axon than in dendrites (Figure 5.3A, line scan inset), suggesting that Stx-YFP is specifically targeted to or retained at the axonal membrane. To understand the identity of syntaxin-1 vesicles in developing hippocampal neurons we co-expressed the synaptic vesicle protein synaptophysin-mCherry with syntaxin-1-EYFP. Synaptophysin-mCherry puncta (Figure 5.3B, arrowheads) were located in both the axon and the dendrites, confirming previous results (Cameron et al., 1991). The majority of syntaxin-1-EYFP and synaptophysin-mCherry puncta co-localized in axon and dendrites (Figure 5.3C, arrowheads). Together, these suggest that syntaxin-1-EYFP is localized on synaptic precursor vesicles together with synaptophysin-mCherry in dendrites and axons.

5.2.4 A METHOD FOR TRACKING VESICLES IN TIME LAPSE IMAGING

Next, we analyzed vesicle movement over time in living neurons. Neurons that expressed syntaxin-1-EYFP at 5 and 6 DIV (Figure 5.3A) were incubated with BPB/ NH_4Cl (pH 7.4) for two minutes (as in Figure 5.1B) to visualize syntaxin-1-EYFP vesicles (Figure 5.4B, arrowheads). Coordinates, with sub-pixel resolution, of manually selected vesicles were determined in each frame and recorded automatically using MTrackJ (Meijering et al., 2012). A colored line through the coordinates of previous frames created a trace depicting movement of the vesicle through the neuronal processes (Figure 5.4C). These coordinates were used to calculate the distance moved per time interval (Figure 5.4D), vesicle velocity, total distance moved, and distance moved per moving period. Directionality (anterograde or retrograde) was determined by calculating the relative displacement towards a marker placed in the image closest to the soma (Figure 5.4C, *). A moving vesicle was defined by a minimum displacement of 150 nm (Figure 5.4D, white dashed line) in two consecutive frames/time intervals. Vesicles that left behind a trace after tracking were also well above this threshold (Figure 5.4C and D, example 4) and vesicles that did not (Figure 5.4C and D, example 3 and 5) remained almost always below this threshold. We used this method to investigate the role of FEZ1 phosphorylation in the transport of syntaxin-1 vesicles in developing hippocampal neurons in culture.

5.2.5 MAJORITY OF SYNTAXIN-1 VESICLES ARE MOBILE AND DISPLAY DIRECTIONAL MOVEMENT

FEZ1 is a kinesin-1 family member KIF5C adaptor protein involved in the transport of synaptic vesicles (Toda et al., 2008), mitochondria (Fujita et al., 2007) and syntaxin-1/Munc18 transport complexes (Chua et al., 2012). FEZ1 links syntaxin-1a and Munc18 to the motor protein kinesin and binds Munc18 in a phosphorylation-dependent manner, which may mediate loading/unloading of syntaxin trafficking vesicles to KIF5C in C. elegans (Chua et al., 2012). A phosphorylation-deficient mutant of FEZ1, FEZ1 S58A, aggravates syntaxin clustering in a UNC-76 (FEZ1 ortholog) mutant C. elegans (Chua et al., 2012). Here, we investigated the role of FEZ1 phosphorylation in the transport of syntaxin-1 vesicles in developing hippocampal neurons in culture. For this, we transfected syntaxin-1-EYFP and N-terminal V5-epitope tagged FEZ1 wild-type (WT) or FEZ1 S58A (Figure 5.5E) at 4 and 5 DIV and analyzed syntaxin-1-EYFP vesicle transport 24 hours later in all neurites and in the axon using 2-minute time lapse imaging in combination with BPB/NH4Cl (pH 7.4) application. The majority of vesicles (81 %) in S58A expressing neurons were classified as moving vesicles, similar to vesicles in FEZ1 WT (76 %) (Figure 5.5A, left panel). Also in axons, vesicles showed similar mobility (Figure 5.5A, right panel). The majority of moving vesicles switched directionality at least once

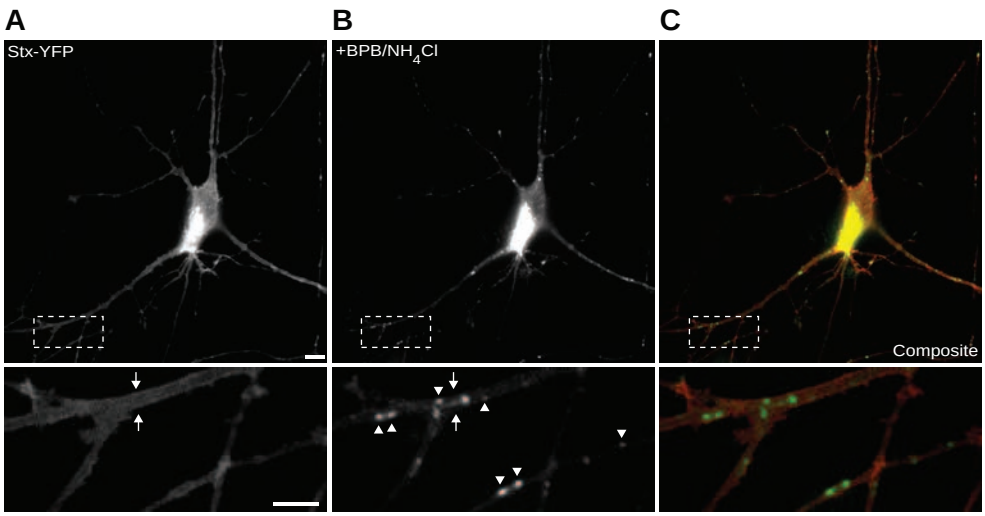


Figure 5.2 Developing hippocampal neurons express syntaxin-1-YFP on plasma membrane and vesicles
Developing hippocampal neuron in culture expressing syntaxin-1-EYFP before (A) and during bromophenol blue/NH4Cl application (B) and shown as composite in (C). A, Syntaxin-1-EYFP (Stx-YFP) expressed in a developing hippocampal neuron in culture results in global fluorescence throughout neuron (top panel). A magnification (bottom panel) of a neuronal process (white rectangle in top panel) shows global fluorescence with enrichment at the plasma membranes (arrows). Scale bar top panel 10 µm, bottom and middle panel 5 µm. B, Superfusion of Bromophenol blue (BPB) and NH4Cl (pH 7.4) results in punctate fluorescence in soma and neuronal processes. Magnification shows vesicular Stx-YFP (arrowheads) in neuronal processes during BPB/ NH4Cl (pH 7.4) application. Note that membrane fluorescence has decreased (arrows). C, Composite of plasma membrane Stx-YFP (from A, red) and vesicular Stx-YFP (from B, green). Stx-YFP, syntaxin-1-EYFP; BPB, bromophenol blue.

(Figure 5.5B), which in dendrites would not require switching to dynein motors (Baas et al., 1988). The majority of axonal vesicles also displayed bidirectional transport, suggesting that these vesicles switched between kinesin and dynein driven transport (Figure 5.5B, right panel). Interestingly, no vesicles were found to move in anterograde direction in FEZ1 S58A neurons (Figure 5.5B). However, we did not find a significantly altered distribution in transport directions after FEZ1 S58A (Figure 5.5B), likely due to the small number of anterograde moving vesicles in control. Hence, FEZ1 S58A overexpression does not inhibit retrograde and bi-directional transport of syntaxin-1-EYFP vesicles. The lack of vesicles moving in the anterograde direction is striking but requires further investigation with larger cell numbers. Next, we analyzed vesicle velocity. Bidirectional vesicles moved with average 151 ± 12 nm/s in FEZ1 S58A expressing neurons, which was similar to average 134 ± 6 nm/s in FEZ1 WT expressing neurons (Figure 5.5C). Surprisingly, anterograde vesicles, moving at ~500 nm/s, were significantly faster than bidirectional vesicles (Figure 5.5C). However, we did not find a difference between FEZ1 WT and S58A neurons. The average distance moved of a vesicle (not accounting for direction) per moving period was also unchanged, vesicles in FEZ1 WT neurons traveled on average 1853 +/- 626 nm per moving period (Figure 5.5D). Similar results were found in axons. Together, this shows that expression of a putative dominant-negative FEZ1 does not lead to a complete block of syntaxin-1 transport in developing hippocampal neurons.

5.2.6 BIDIRECTIONAL VESICLES DISPLAY SIMILAR MOVEMENT IN BOTH DIRECTIONS

Kinesins in vertebrates move towards the plus end of a microtubule which would direct them in anterograde direction in axons and in a bidirectional direction in dendrites of 7 DIV cultured neurons (Baas et al., 1989). Dyneins move in opposite direction of kinesins (Goldstein and Yang, 2000). This suggests that syntaxin-1 vesicles that display bidirectional transport in axons are sequentially transported by kinesins and dyneins (Koushika et al., 2004). Therefore, we analyzed the anterograde and retrograde component of bidirectional transport to investigate if the anterograde direction was inhibited by FEZ1 S58A expression. Retrograde velocity of all vesicles in FEZ1 S58A expressing neurons was indistinguishable from vesicles in FEZ1 WT expressing neurons. Anterograde velocity of all vesicles in FEZ1 S58A expressing neurons was also unaltered (Figure 5.6A, left panel). Microtubule plus ends in dendrites are orientated to both the proximal and distal part of the dendrite in a ratio of ~50 %. Therefore we focused on the axons, where plus ends are all orientated towards the distal part. As expected, retrograde velocity of axonal vesicles was unchanged by FEZ1 S58A expression as dyneins are assumed to be responsible for retrograde transport. However, anterograde velocity was also unchanged in axonal vesicles (Figure 5.6A, right panel). Additionally, the distance moved and time spent moving per vesicle (all or axonal) was not different between neurons expressing WT or mutant FEZ1 (Figure 5.6B and C). Together, these results suggest that phosphorylation of serine 58 of FEZ1 is not essential for bidirectional transport of syntaxin-1-EYFP in axons.

5.3 DISCUSSION

In this study we investigated the role of fasciculation and elongation protein-ζ1 (FEZ1) in the transport of syntaxin-1 in developing hippocampal neurons. We developed a novel method to simultaneously quench plasma membrane syntaxin-1-EYFP and neutralize vesicular pH to reveal vesicular syntaxin-1-EYFP. Syntaxin-1-EYFP vesicles were present in dendrites and axons and co-localized with synaptophysin-mCherry. Our study focused on the effect of phosphorylation of FEZ1

on transport of syntaxin-1-EYFP vesicles, since a phosphorylation-deficient mutant FEZ1 expressed in FEZ1 deficient C. elegans aggravated syntaxin transport defects. Phosphorylation-deficient mutant FEZ1, FEZ1 S58A, expressed in cultured hippocampal neurons decreased neither the number of mobile vesicles, nor the average velocity of mobile vesicles. Vesicles that changed direction during the course of imaging moved with similar speed and reached similar distances in both directions, irrespective of the phosphorylation status of FEZ1. Thus, we were able to distinguish vesicular syntaxin-1 from plasma membrane syntaxin-1 and analyze speed and direction of vesicular syntaxin-1. However, FEZ1 S58A over-expression did not alter the trafficking characteristics of syntaxin-1 vesicles, indicating that FEZ1 S58A does not act as a dominant negative on transport of syntaxin-1.

To test if phosphorylation of FEZ1 in rodents is essential, future experiments should express FEZ1 WT and FEZ1 S58A on fez1 null mutant background.

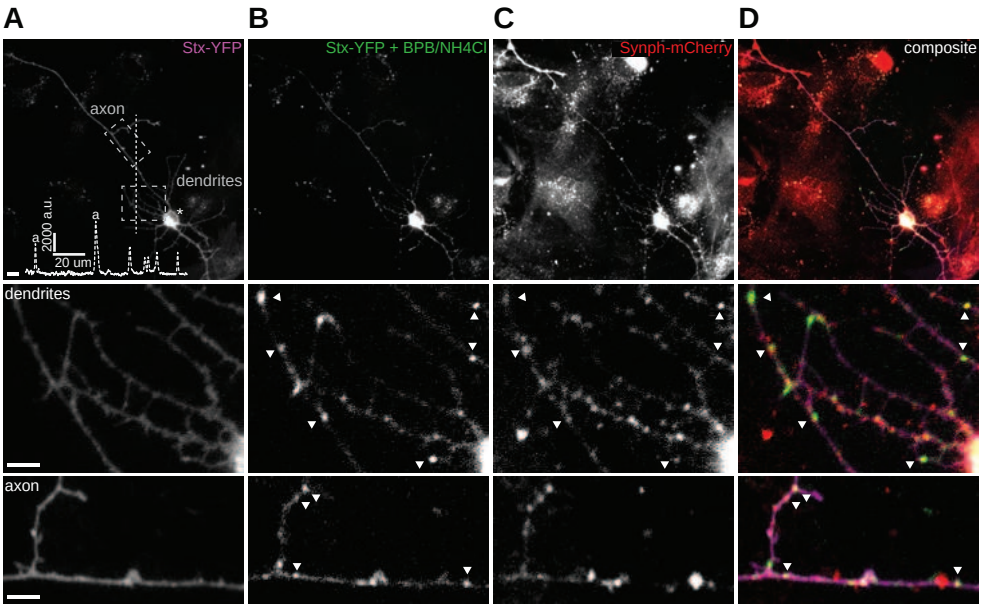


Figure 5.3 Vesicular syntaxin-1-EYFP colocalizes with synaptophysin-mCherry in dendrites and axons
Developing hippocampal neuron in culture expressing syntaxin-1-EYFP before (A) and during bromophenol blue/NH4Cl application (B), co-expressing synaptophysin-mCherry (C) shown as composite in (D). A, Syntaxin-1-EYFP (Stx-YFP) fluorescence in soma (*), dendrites and the axon. Note that fluorescence in soma is highest in perinuclear region, and that fluorescence in axon is higher than in dendrites (see line scan of vertical line; a, axons; unmarked peaks, dendrites). Rectangles indicate zoomed regions of dendrites and axon in middle and bottom panel, respectively. Scale bar top panel 10 µm, bottom and middle panel 5 µm. B, Stx-YFP fluorescence during bromophenol blue (BPB) and NH4Cl (pH 7.4) application is punctate throughout the neuron. Stx-YFP vesicles (arrowheads) are located in dendrites (middle panel) and the axon (bottom panel). C, Synaptophysin-mCherry fluorescence is punctate throughout the neuron. Synaptophysin-mCherry vesicles (arrowheads) are located in dendrites (middle panel) and the axon (bottom panel). D, Synaptophysin-mCherry (red) generally colocalizes with Stx-YFP (green) in vesicles in dendrites (middle panel) and the axon (bottom panel). Plasma membrane Stx-YFP (magenta) is located throughout the neuron.

5.3.1 SYNTAXIN-1 IS EXPRESSED ON VESICLES AND THE PLASMA MEMBRANE OF DEVELOPING NEURONS

Expression of syntaxin-1-EYFP resulted in global fluorescence throughout the neurons. Application of a hydrophilic quencher, bromophenol blue, with NH_4Cl (pH 7.4) resulted in decrease of the overall fluorescence and appearance of fluorescent puncta, indicating that syntaxin-1-EYFP was expressed at the plasma membrane and on acidic vesicles in developing neurons. Previous studies in mature neuronal cultures showed that fluorescent syntaxin-1 is expressed at the plasma membrane (Mitchell and Ryan, 2004; Ribrault et al., 2011). In C. elegans, a fluorescently tagged syntaxin ortholog, GFP-UNC-64, showed expression throughout the ventral cords of wild-type worms and a punctate pattern in FEZ1 mutant worms (Chua et al., 2012). Other studies in developing neuronal cultures showed that fluorescent syntaxin-1 moved in puncta positive for Bassoon along microtubules of neuronal processes (Su et al., 2004). This suggests that after the delivery of vesicles at target sites, possibly growth cones (Chua et al., 2012) or newly formed synapses (Cai et al., 2007), syntaxin-1 is inserted in the plasma membrane where it can diffuse laterally to other parts of the neuronal process (Mitchell and Ryan, 2004; Ribrault et al., 2011). Depending on the developmental state of the neuron the amount of plasma membrane syntaxin-1 might vary. Syntaxin-1 was enriched in axons, in line with previous results (Garcia et al., 1995; Mitchell and Ryan, 2004; Ribrault et al., 2011) suggesting a specific targeting of syntaxin-1 positive vesicles to axons or retention of plasma membrane syntaxin-1 at the axon after insertion. We did not find evidence of a specific targeting of vesicles to axons (Figure 5.3). Indeed, many axonal membrane proteins are initially transported to membranes of axons and dendrites, and become enriched in axons after specific endocytosis at dendritic membranes (Garrido et al., 2001; Sampo et al., 2003; Wisco et al., 2003). Additionally, membrane proteins may be specifically retained at the axon via a diffusion barrier at the axon hillock (Winckler et al., 1999). Together, our results indicate that early in development syntaxin-1 can be found at transport vesicles and the plasma membrane, which suggests that active vesicular transport of syntaxin-1 and passive lateral diffusion of syntaxin-1 along the plasma membrane can occur at the same time during development.

5.3.2 SYNTAXIN-1 IS TRANSPORTED ON SYNAPTIC VESICLE PRECURSORS

Syntaxin-1-EYFP vesicles were located in dendrites and axons of developing neurons. Synaptophysin is an integral synaptic vesicle (SV) protein (Evans and Cousin, 2005; Valtorta et al., 2004), and is found in axons and dendrites in developing neurons (Cameron et al., 1991) and in synapses of mature neurons (Jahn et al., 1985). Synaptophysin is transported together with synaptotagmin and Rab3A on synaptic vesicle precursors by kinesin family member KIF1A. However, these vesicles from rat spinal nerves did not contain syntaxin-1a (Okada et al., 1995). Surprisingly, the majority of syntaxin-1-EYFP vesicles co-localized with synaptophysin-mCherry (Figure 5.3). Syntaxin-1 is transported via other kinesin family members, KIF5B and KIF5C (Chua et al., 2012; Su et al., 2004), than synaptophysin. Syntaxin-1 is generally believed to be transported on piccolo-bassoon transport vesicles (PTVs) that transport active zone components to new synapses (Shapira et al., 2003; Zhai et al., 2001), while synaptophysin is transported on synaptic protein transport vesicles (STVs) (Nakata et al., 1998; Smith et al., 2000). However, syntaxin-1 or synaptophysin may be transported by more than one kinesin family member: mitochondria are transported by at least two different family members, KIF1B and KIF5 (Hirokawa et al., 2010;

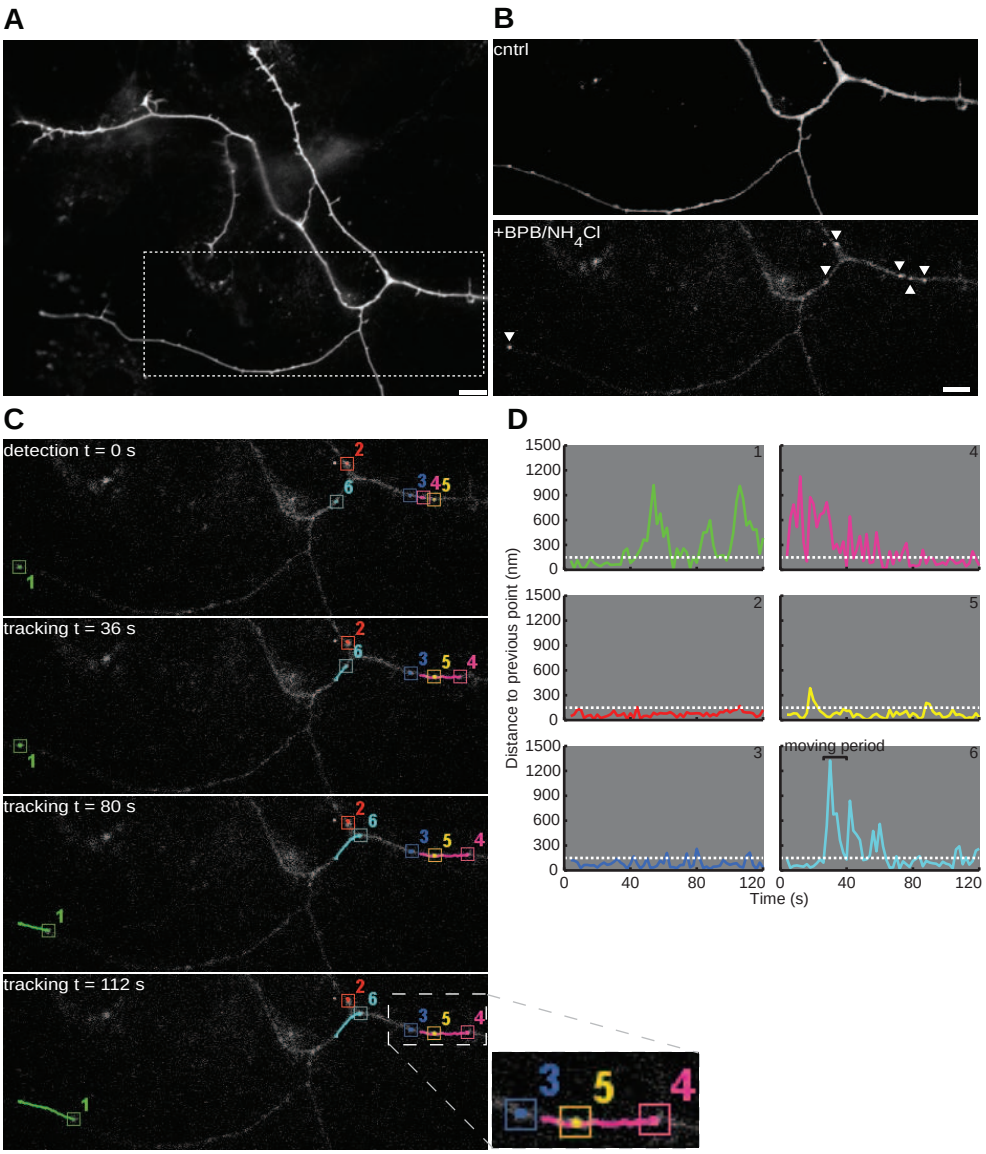


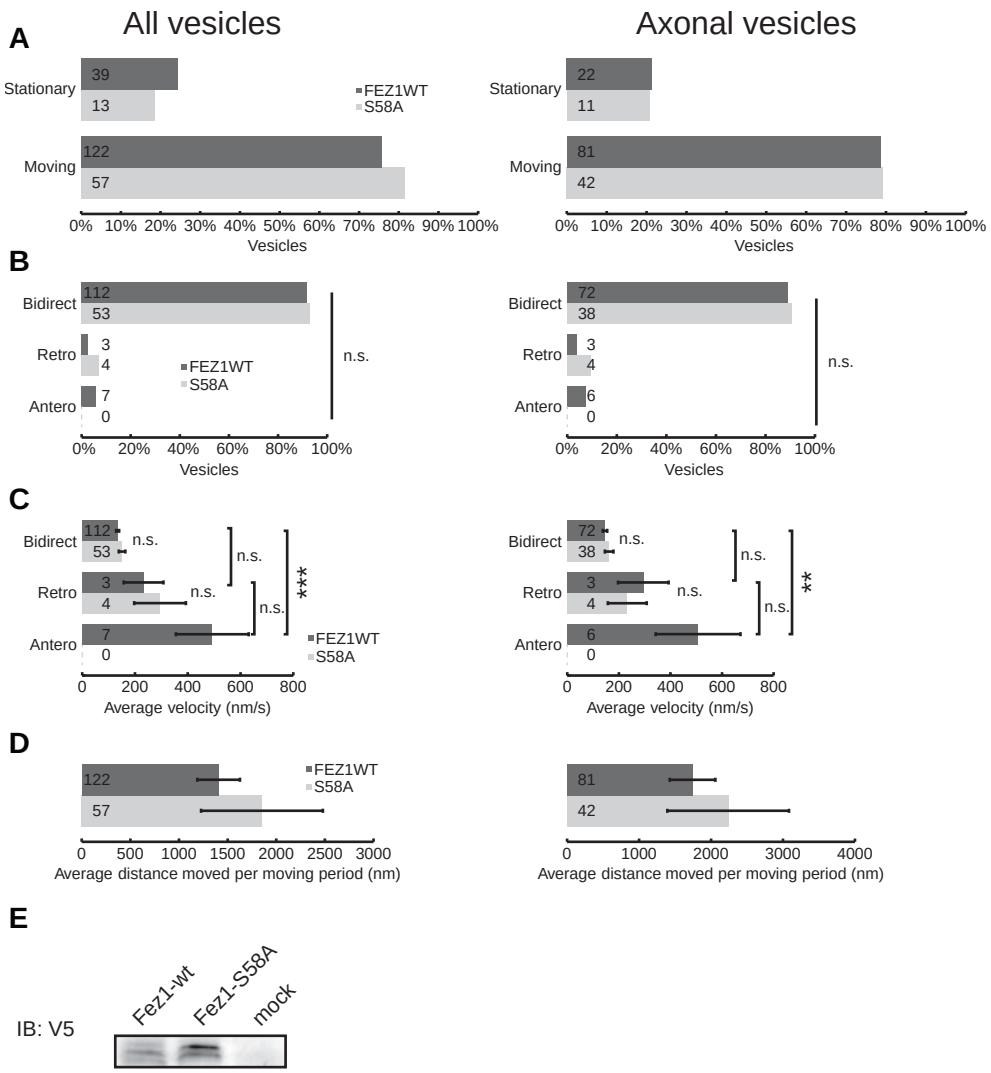
Figure 5.4 Analysis of vesicle transport A, Syntaxin-1-EYFP (Stx-YFP) expressed in a developing hippocampal neuron in culture results in global fluorescence throughout a distal neuronal process. B, A magnification of the rectangle area in A during Tyrode's application (cntrl) and during bromophenolblue (BPB)/ NH4Cl (pH 7.4) application. Several puncta (arrowheads) appear after BPB/ NH4Cl application. C, Coordinates of a punctum are extracted in MTrackJ by determining the intensity-weighted mean position of a 9x9 pixel ROI placed over the punctum in each frame. Example images show initial detection at t = 0 s of six puncta and further tracking at several time points (tracking t = 36 s, 80 s, 112 s). Note that tracks of moving puncta are depicted by a colored line. D, The coordinates of a punctum in two sequential time points is used to calculate the distance traveled per time point (distance to previous point). From this parameter other parameters are derived (velocity, distanced moved). A moving punctum is defined by at least two sequential time points with more than 150 nm movement per point (example 1, 4, 5, 6). A moving period is defined by at least two sequential time points with more than 150 nm movement per point in between time points with less than 150 nm movement.

Hirokawa et al., 2009). Our results suggest that syntaxin-1 and synaptophysin are transported together on synaptic vesicle precursors in developing hippocampal cultures. Alternatively, syntaxin-1 and synaptophysin could be expressed on separate vesicles that are transported together in clusters (Bury and Sabo, 2011). In a future study, immunohistochemistry may corroborate the hypothesis that endogenous syntaxin-1 and synaptophysin are transported together.

5.3.3 SYNTAXIN-1 VESICLES ARE TRANSPORTED BIDIRECTIONALLY OVER SHORT DISTANCES

The majority of syntaxin-1 vesicles were mobile and moved in both anterograde and retrograde direction during 2 minutes of imaging. Average speeds for bidirectional vesicle transport were relatively slow compared with axonal transport (Vallee and Bloom, 1991) although such speeds have been reported before (Bury and Sabo, 2011). Bidirectional vesicles likely have plus-end and minus-end oriented motors simultaneously attached and these opposing motors either alternate in activity or engage in a so-called tug-of-war (Franker and Hoogenraad, 2013; Welte, 2004), where kinesins would pull the vesicles towards the plus-end of the microtubules and dyneins towards the minus-end. This tug-of-war of motors can move vesicles into one direction if one motor prevails, although we observed that most bidirectional vesicles remained within 1 µm of their original position. A minority (≤ 10 %) moved unidirectionally, either in anterograde or retrograde direction. We did not find anterograde moving vesicles in FEZ1 S58A expressing neurons: this might suggest an effect of FEZ1 S58A on anterograde transport. However, the distribution of directions we observed was not significantly changed in FEZ1 S58A expressing neurons due to the low number of anterograde vesicles. Anterograde vesicles were significantly faster than bidirectional vesicles. This suggests that bidirectional vesicles undergo pulling forces from kinesins and dyneins simultaneously, in line with a tug-of-war, resulting in a reduced net velocity. Alternatively, vesicle traces typically show an acceleration period leading up to a top speed, followed by deceleration period (Figure 5.4D); bidirectional vesicles switch direction and therefore have more acceleration/deceleration periods resulting in slower average speeds. Together, the majority of syntaxin-1 vesicles are transported over short distances without a preference for direction. This result supports a model where syntaxin-1 is transported into neurites up to certain distances, not necessarily to the distal end, and later is inserted into the membrane. Whether the insertion sites are new synapses remains to be tested.

Figure 5.5 Role of FEZ1 on syntaxin-1-EFYP vesicle trafficking in developing hippocampal cultures A, After FEZ1 wild-type (WT) overexpression, 76% of all syntaxin-1-EFYP vesicles (left panel) were mobile at one or more points during the time lapse recordings, compared with 81% after FEZ1 S58A overexpression. 79% of axonal syntaxin-1-EYFP vesicles (right panel) were mobile in FEZ1 WT and S58A expressing neurons. B, Six percent of all mobile vesicles (left panel) moved in anterograde direction (Antero), 2% moved in retrograde direction (Retro) and 92% displayed bidirectional movement after FEZ1 WT overexpression, compared with 0% anterograde, 7% retrograde, and 93% bidirectional movement after FEZ1 S58A overexpression (difference between FEZ1 WT and S58A in number of vesicles per category, Fisher's exact test: n.s., $p>0.05$). Seven percent of all mobile axonal vesicles (right panel) moved in anterograde direction (Antero), 4% moved in retrograde direction (Retro) and 89% displayed bidirectional movement after FEZ1 WT overexpression, compared with 0% anterograde, 10% retrograde, and 90% bidirectional movement after FEZ1 S58A overexpression (difference between FEZ1 WT and S58A in number of vesicles per category, Fisher's exact test: n.s., $p>0.05$). >>>



>>> C, Anterograde vesicles (Antero) moved with 493 ± 138 nm/s (axonal: 507 ± 164 nm/s), retrograde vesicles (Retro) with 232 ± 75 nm/s (axonal: 232 ± 75 nm/s), and bidirectional vesicles (Bidirect) with 134 ± 6 nm/s (axonal: 146 ± 8 nm/s) in FEZ1 WT neurons. In FEZ1 S58A neurons, retrograde vesicles moved with 295 ± 98 nm/s (axonal: 295 ± 98 nm/s) and bidirectional vesicles with 151 ± 12 nm/s (axonal: 163 ± 17 nm/s). Note that no anterograde vesicles were found in FEZ1 S58A neurons. (FEZ1 WT vs S58A per direction, Mann-Whitney: non-significant (n.s.), p>0.05. Velocity in 3 directions compared in FEZ1 WT, Kruskal-Wallis: n.s., p>0.05; ***, p<0.001). D, Average distance trafficked in moving periods. Moving periods were defined as being in between two non-moving/static periods. Vesicles in FEZ1 WT neurons trafficked on average 1407 ± 219 nm (axonal: 1743 ± 317 nm) and 1853 ± 626 nm (axonal: 2238 ± 845 nm) in FEZ1 S58A neurons. Data plotted as means ± SEM (error bars); n.s., non-significant. E, Western blot analysis of protein expression of V5-tagged FEZ1 WT, FEZ1 S58A and mock transfection in HEK cells detected with V5 antibody.

5.3.4 PHOSPHORYLATION OF SERINE 58 OF FEZ1 IS FUNCTIONALLY REDUNDANT IN DEVELOPING NEURONS

Syntaxin is transported via adaptor protein syntabulin in rodents and adaptor protein FEZ1 in nematodes (Chua et al., 2012; Su et al., 2004). FEZ1 co-localizes with syntaxin-1 on tubulin tracts of growth cones of developing rat neurons, which suggests that FEZ1 also acts as an adaptor protein for syntaxin-1 transport in rodents. To test this hypothesis we made use of a mutant FEZ1, FEZ1 S58A, which cannot be phosphorylated on serine 58. This mutant aggravates syntaxin-1 vesicle clustering in C. elegans when expressed on null mutant background (Chua et al., 2012). Because, FEZ1 binds syntaxin-1 independent of phosphorylation, but FEZ1 S58A is unable to bind to kinesin-1 and Munc18, FEZ1 S58A may act as a dominant negative mutant of FEZ1. However, we found that expression of FEZ1 S58A in wildtype neurons neither decreased the number of moving vesicles nor decreased the velocity of vesicles in the whole neuron and the axon. Additionally, we analyzed anterograde and retrograde components of bidirectional transport, but found that FEZ1 S58A did not have an effect on anterograde transport. Thus, phosphorylation of serine 58 of FEZ1 may not have similar functions in worms and rodents. However, the FEZ1 region responsible for Munc18 binding is evolutionary conserved (Assmann et al., 2006), human FEZ1 (S58A) is functional in worms and the S58A mutation prevents binding to human Munc18 domain 3 (Chua et al., 2012), indicating that phosphorylation of S58 is functionally important in mammals. In contrast to C. elegans, in humans and rodents FEZ1 has a homologue, FEZ2. Human and rat FEZ2 have 50% and 48% sequence identity with human and rat FEZ1, respectively. Additionally, FEZ2 has similar functionality in PC12 cells, and is expressed ubiquitously, including the hippocampus, and abundantly throughout the mouse embryonic stages (Toshitsugu et al., 2004). Moreover, human FEZ2 interacts with the same proteins as FEZ1 in a yeast two-hybrid system (Alborghetti et al., 2011) and FEZ1 knock-out mice do not exhibit obvious abnormal brain architecture (Sakae et al., 2008). Ubiquitously expressed FEZ2 may compensate for the lack of neuronal FEZ1. However, when FEZ1 S58A is expressed in a normal FEZ1 and FEZ2 background it should still bind syntaxin-1 without being able to bind KIF5C, thereby hijacking syntaxin-1 from transport complexes, unless FEZ1 S58A is not expressed to sufficient amounts to effectively block all the syntaxin-1 on a vesicle. A second adaptor protein, syntabulin, interacts with syntaxin-1A, via its carboxyl coiled-coil domain, and mediates the transport of syntaxin-1 to neuronal processes via the kinesin family member 5B (KIF5B) (Su et al., 2004). It is unknown to what domain of syntaxin-1 FEZ1 binds. Syntabulin may bind to a different region on syntaxin-1 without being hampered by FEZ1 S58A. Moreover, depletion of syntabulin impairs the proper distribution and trafficking of syntaxin-1 in rat hippocampal culture, suggesting an essential role for syntabulin in syntaxin-1 trafficking. Our results support that hypothesis.

5.4 EXPERIMENTAL PROCEDURES

5.4.1 NEURONAL CELL CULTURE AND TRANSFECTION

Dissociated hippocampal cultures were obtained from E18 wild-type mice as described previously (Meijer et al., 2012). In brief, hippocampi were dissected in HEPES buffered HBBS (Invitrogen) and digested with 0.25 % trypsin (Invitrogen) at 37° C for 20 min. After washing and trituration, cells were plated at a density of 25,000 cells/well for low-density cultures on top of a pre-grown rat glia feeder layer on 18 mm coverslips. Cultures were kept in Neurobasal medium (containing 2 % B27,

18 mM HEPES, 0.5 mM GlutaMAX, and penicillin/streptomycin; all obtained from Invitrogen), and half the medium was replaced once every week in low-density cultures.

For imaging experiments on wild-type neurons, syntaxin-1-EYFP, FEZ1WT and FEZ1-S58A were transfected using calcium phosphate precipitate at 6-10 DIV as described previously (Köhrmann et al., 1999).

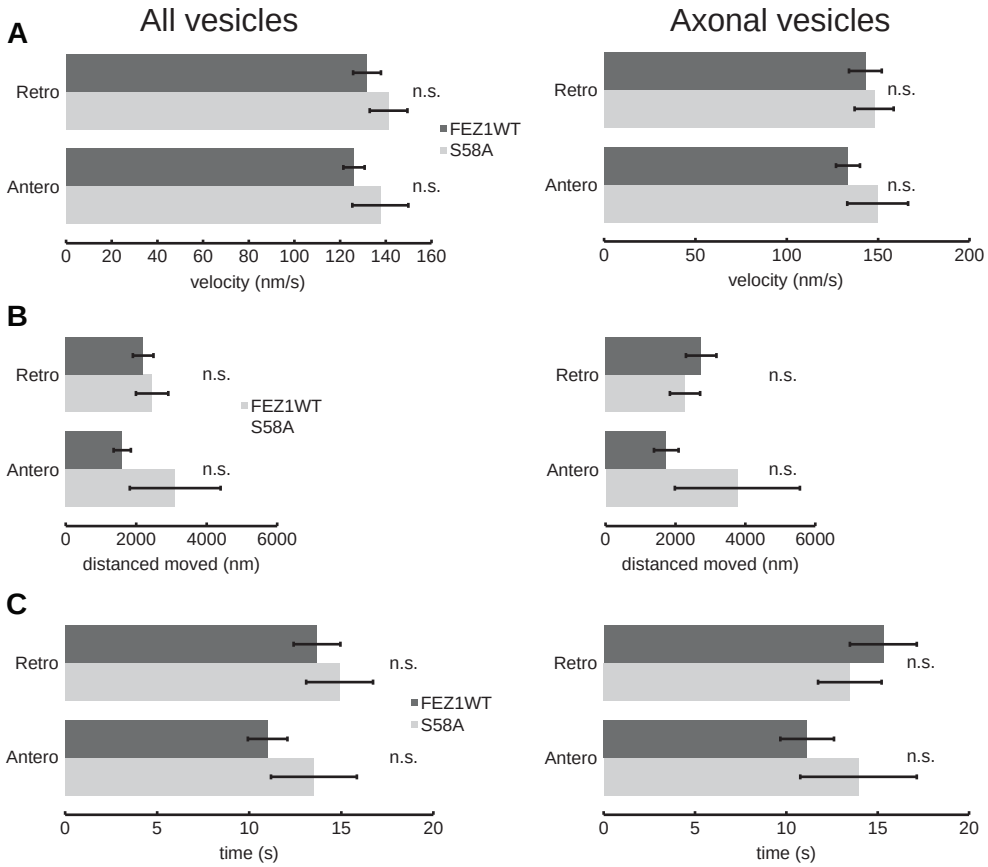


Figure 5.6 Comparison of anterograde and retrograde component of bidirectional vesicles A, In FEZ1 WT and FEZ1 S58A neurons bidirectional vesicles moved in anterograde direction with 126 ± 5 nm/s and 138 ± 12 nm/s (axonal: 133 ± 7 nm/s and 150 ± 17 nm/s), respectively, and in retrograde direction with 132 ± 6 nm/s 141 ± 8 nm/s (axonal: 143 ± 9 nm/s and 148 ± 11 nm/s), respectively (FEZ1 WT vs S58A per direction, Mann-Whitney: non-significant (n.s.), p>0.05.). B, In FEZ1 WT and FEZ1 S58A neurons bidirectional vesicles moved in anterograde direction a total of 1604 ± 245 nm and 3107 ± 1287 nm (1739 ± 352 nm and 3772 ± 1788 nm), respectively, and in retrograde direction a total of 2197 ± 293 nm and 2451 ± 458 nm (2734 ± 436 nm and 2270 ± 433 nm), respectively (FEZ1 WT vs S58A per direction, Mann-Whitney: non-significant (n.s.), p>0.05). C, In FEZ1 WT and FEZ1 S58A neurons bidirectional vesicles moved in anterograde direction a total of 11 ± 1 s and 14 ± 2 s (axonal: 11 ± 1 s and 14 ± 3 s), respectively, and in retrograde direction a total of 14 ± 1 s and 15 ± 2 s (axonal: 15 ± 2 s and 13 ± 2 s), respectively. (FEZ1 WT vs S58A per direction, Mann-Whitney: non-significant (n.s.), p>0.05.). Data plotted as means ± SEM (error bars).

5.4.2 CONSTRUCTS

Syntaxin-1-EYFP has been described before (Toonen et al., 2005) and is full length rat Syntaxin1a in pEYFP-N1 (CMV promoter, Clontech). Synaptophysin-mCherry (P38-mCherry, pcDNA3.1) was a kind gift from A. Jeromin (Allen Brain Institute, Seattle, WA), mCherry inserted at AA184 (intravesicular loop, same place as pHluorin in SyPhy)(Granseth et al., 2006). FEZ1 WT and FEZ1 S58A have been described before (Chua et al., 2012) and are full length human FEZ1 (wild-type or mutated) in pcDNA3.1-nV5-DEST (Invitrogen).

5.4.3 LIVE IMAGING

All live cell imaging experiments were conducted on a custom-built Tandem Illumination Microscope (TIM, Olympus) consisting of an inverted imaging microscope (IX81) and an upright laser scanning microscope. The inverted microscope part was used for imaging fluorescence, using an MT20 light source (Olympus), appropriate filter sets (Semrock) and a 60x oil objective (NA 1.49) with or without a 1.6x additional magnification, on a Hamamatsu EM-CCD camera (C9100-02; Hamamatsu City, Japan). Xcellence RT imaging software (Olympus) was used for controlling the microscope and recording the images. Coverslips with neurons were placed in an imaging chamber and perfused with imaging solution (Tyrode's: 2 mM CaCl2, 2.5 mM KCl, 119 mM NaCl, 2 mM MgCl2, 20 mM glucose, and 25 mM Hepes, pH 7.4).

Bromophenol Blue/NH4Cl (pH 7.4) application. Bromophenol Blue (BPB) was dissolved to a final concentration of 0.25 mM into standard Tyrode's solution or Tyrode's solution to neutralize vesicular pH (where 50 mM NaCl was replaced by 50 mM NH4Cl, pH 7.4). BPB solutions were let to dissolve properly for 1 hour followed by filtration through a 0.2 µm filter to remove undissolved particles. BPB solutions were applied locally using a barrel pipette for 2 minutes followed by washing with standard Tyrode's.

5.4.4 IMAGE ANALYSIS

Image stacks from time-lapse recordings were loaded into ImageJ (NIH, USA) and vesicles that appeared after BPB/NH4Cl application were manually tracked using the MTrackJ plugin (www.imagescience.org/meijering/software/mtrackJ)(Meijering et al., 2012). Generated files containing vesicle coordinates were further analyzed using custom written programs in Matlab (MathWorks) to extract shown parameters.

5.4.5 STATISTICS

Differences between two groups were tested for significance using a Student's t test for unpaired data when data passed a normality test (Kolmogorov-Smirnov) and a Mann-Whitney (M-W) test when not. Differences between more than two groups were tested for significance using a Kruskal-Wallis (K-W) test, and pairwise comparisons using M-W tests were used where applicable. P-values were adjusted using a False Discovery Rate (FDR) correction (Benjamini and Hochberg, 1995) for number (#) of multiple comparisons, in short FDR (#) corrections. For testing significant differences in amount of vesicles per group the Fisher's exact test was used. Differences were regarded significant when $p < 0.05$. Statistics were performed in SPSS (IBM). All data plotted as mean ± SEM (error bars or shaded area).

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